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Functional Domains of Pseudomonas Exotoxin Identified by Deletion Analysis of the Gene Expressed in E. Coli

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Summary

Pseudomonas exotoxin A is a single chain toxin with three structural domains that inhibits protein synthesis in eukaryotic cells by catalyzing ADP ribosylation of elongation factor 2. To study the function of these domains, we deleted different portions of the PE structural gene and expressed these constructs in E. coli using an inducible T7 promoter. These studies indicate that structural domain Ia is required for cell recognition, that structural domain II is required to translocate the toxin across a cellular membrane, and that structural domain III and a portion of domain Ib are required for ADP ribosylation activity. Toxin lacking domain Ia is about 100-fold less toxic to mice than intact PE and should be a useful molecule for the construction of immunotoxins.

Introduction

Toxins are extremely potent cell killing agents that are responsible for many human diseases. Because of their high activity, these agents have been attached to monoclonal antibodies producing specific cytotoxic agents (immunotoxins) that may be useful in cancer therapy (Trowbridge and Domingo, 1981; Tsukada et al., 1982; Vitetta et al., 1983; Pastan et al., 1986). Pseudomonas exotoxin A (PE), which is secreted by Pseudomonas aeruginosa, makes an extremely active immunotoxin (IT) when conjugated to an antibody (FitzGerald et al., 1983a, 1983b). To construct PE-containing immunotoxins, native PE is reacted with iminothiolane. This reaction serves two purposes. It introduces two new sulfhydryl groups which are used for coupling antibody to the toxin. Derivatization by iminothiolane also decreases the binding of PE to the PE receptor and thereby minimizes undesirable side effects. Immunotoxins produced in this manner kill human cancer cells in tissue culture (FitzGerald et al., 1983a, 1983b) and in tumor-bearing mice (FitzGerald et al., 1986), but cannot be given in large amounts to animals or patients because of residual binding of the PE-containing immunotoxin to normal liver cells, which causes liver damage (FitzGerald, Willingham, and Pastan, unpublished result). It is clearly desirable to administer as large amounts of immunotoxins as possible to achieve the highest degree of killing of tumor cells. To overcome this reliance on chemical inactivation, we have used recombinant DNA techniques to clone and express at high levels full-length toxin molecules and molecules containing different functional domains of the toxin. In the gene expression system used, the structural gene for PE is fused to a bacteriophage T7 promoter carried on a plasmid, and the T7 RNA polymerase gene, which can transcribe the phage promoter, is carried in the E. coli chromosome and controlled by the bacterial *lac* promoter. PE is produced upon the addition of IPTG (Studier and Moffatt, 1986).

The three-dimensional structure of PE has been determined by x-ray crystallography (Allured et al., 1986). PE contains three structural domains. Domain I contains amino acid residues 1 to 252 (Domain Ia) and 365 to 404 (Domain Ib); domain II contains amino residues 253 to 364; and domain III contains amino acid residues 405 to 613 (Figure 2). Based on the three-dimensional structure of PE, we have constructed plasmids that express different portions of PE. The information obtained from this study identifies the function of these domains and suggests that it should be possible to develop an immunotoxin with high potency and low toxicity selectively using these domains.

Results

Synthesis of Full-Length PE Molecules

Different portions of the structural gene of Pseudomonas toxin were inserted into a T7 expression vector downstream of a ribosome binding site and its accompanying ATG initiation codon (see Figures 1 and 2 and Experimental Procedures). The T7 late promoter, present in these plasmids, is inactive unless T7 RNA polymerase is made from the repressed *lac* promoter by IPTG addition. To produce recombinant proteins, B21(DE3) cells carrying pJH plasmids were grown at 37°C to an A_{650} = 0.3. IPTG was then added at 1 mM to induce T7 RNA polymerase, and incubation continued for 2 hr. pJH1 contained a modified 30 amino acid leader sequence (Figure 2). A second plasmid, pJH2, in which the leader sequence was changed to 21 amino acids and a Met-Lys-Lys-Ile placed at the amino terminus to resemble more closely leader sequences found in E. coli (Benson et al., 1985), was also constructed (Figures 1 and 2). With both plasmids, large amounts of PE proteins were produced and accumulated within the cell (Figure 3, lane 2). However, very little PE protein could be detected secreted into the medium or in the periplasmic space.

We next constructed a clone that encoded a PE molecule without a leader sequence (pJH4) and in which the methionine codon AUG was placed adjacent to the alanine at the amino terminus of the native processed form of PE (Figure 2; Table 1). The protein produced by pJH4 was designated Met-PE. Figure 3, lane 3, shows that a large amount of Met-PE is produced upon induction by IPTG and represents 20% of total cell protein. ADP ribosylating activity equivalent to 0.1 mg of native PE per mg total cell protein was found in the supernatant and 0.2 mg in the pellet (Table 1). These results indicate pJH4 would be a good source from which to prepare large

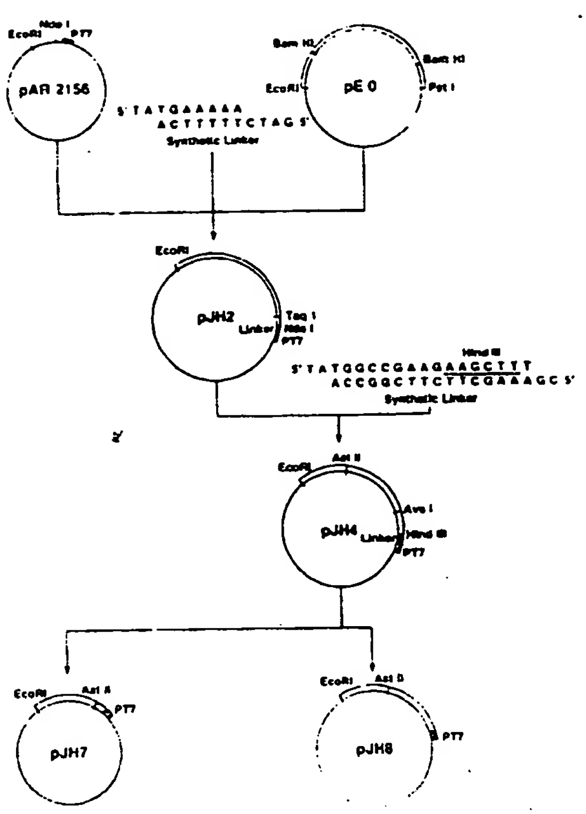

Figure 1. Construction and Structure of Sample Plasmids Used for the Expression of Different Domains of PE

Other plasmids are described in the text. The oligonucleotides used in the construction were synthesized at the National Cancer Institute.

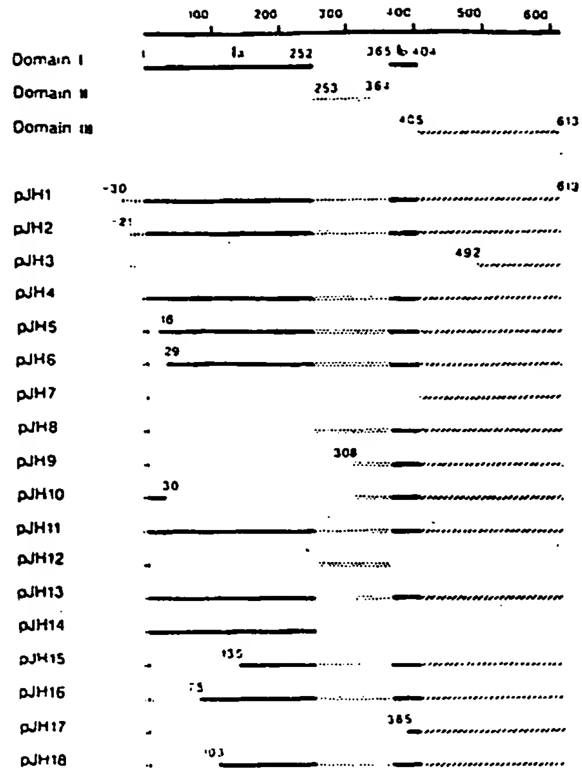

Figure 2. Simplified Map of PE and Its Variant Molecules Made by Recombinant DNA Technics Described in the Text

The map depicts different domains present in each molecule. The end point of each domain or truncated molecule is marked by amino acid number using the first amino acid of mature (processed) native PE as +1.

amounts of PE. This molecule would differ from the native molecule only by the presence of one extra methionine residue at the amino terminus. A small amount of Met-PE was produced without IPTG induction (Figure 3, lane 8), indicating low level expression of T7 RNA polymerase without IPTG induction.

Expression of PE with Various Domains Missing

Using the three-dimensional structure of PE as a guide (Allured et al., 1986), we constructed plasmids expressing different domains of PE. The protein profile of the cells expressing the different constructions was analyzed by SDS gel electrophoresis. The ADP ribosylating activity of the corresponding toxin variants was measured in cell extracts prepared either by sonication or treatment with lysozyme and Triton X-100. Since overproduced proteins often form insoluble aggregates, the extracts were centrifuged at 12,000 × g and the pellet dissolved in 8 M urea and diluted into assay buffer. The amount of recombinant protein accumulated was variable, probably because of differences in degradation of the different size proteins produced even though a protease deficient strain was employed (see Experimental Procedures). Usually a new protein band could be detected by SDS gel electrophoresis and Coomassie blue staining, and enzymatic activity could be measured in cell extracts, but the presence of a modified form of PE was also demonstrated by immunoblotting using a polyclonal antibody to PE. All the constructions shown in Table 1 and Figure 2 produce a toxin variant that is distinguished by one or more of these properties.

A few PE derivatives were selected for more extensive study because they had deletions encoding relevant and interesting portions of the PE molecule and because they produced sufficient protein to allow further study (Table 2). Of particular interest is pJH13, which has a deletion of the first half of domain II and produces a polypeptide containing full domain I, half of domain II, and entire domain III (62 kd). pJH14, which has a deletion of domain II, Ib, and III produces only domain Ia (27 kd). pJH7 has a deletion of domain I and II and produces domain III (23 kd). pJH17 produces a polypeptide with domain III and an adjacent portion of domain Ib (26 kd). And pJH8, which has a deletion of domain Ia, produces a polypeptide encompassing domain II, Ib, and III (40 kd).

Determination of the Region Containing ADP Ribosylating Activity

pJH8

Clone pJH8 expressed a protein that has a deletion of almost all of domain Ia, retaining only the added methionine and three amino acids at the amino terminus (Figure 2).

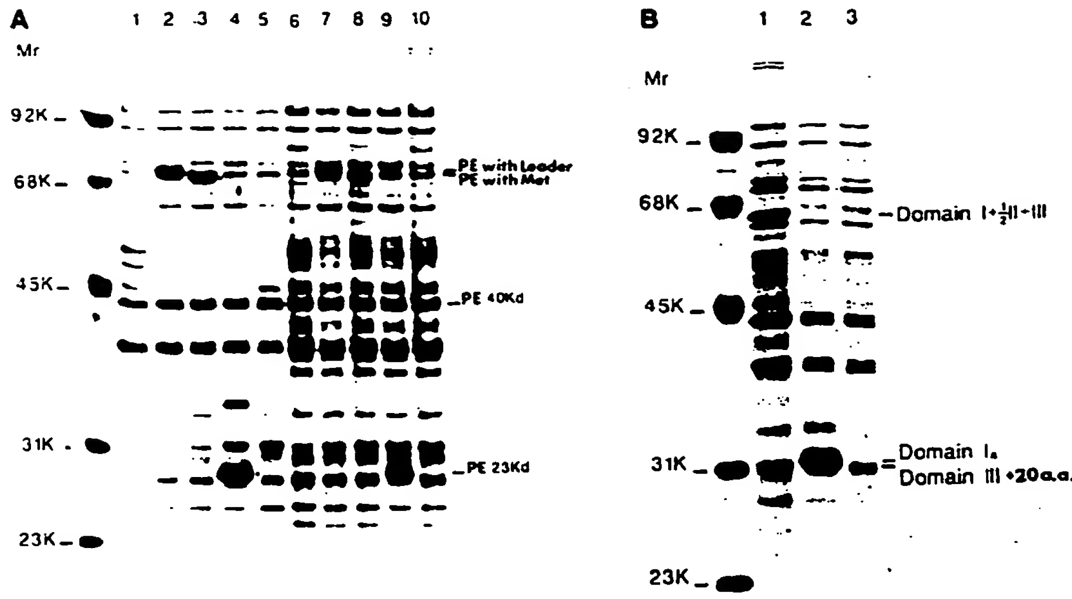

Figure 3. Expression of Some Different PE Constructions in BL21(DE3)

(A) BL21(DE3), BL21(DE3)/pJH2, BL21(DE3)/pJH4, BL21(DE3)/pJH7, and BL21(DE3)/pJH8 were grown in LB medium with or without ampicillin (50 µg/ml). When A_{650} reached 0.3, IPTG was added to a final concentration of 1 mM. Two hr later, cells were harvested and dissolved in Laemmli buffer. Samples (about 20 µg of cellular protein) were heated at 80°C for 5 min and analyzed by 10% SDS-PAGE. Lanes 1 to 5, with IPTG induction; lanes 6 to 10, without IPTG induction; lanes 1 and 6, BL21(DE3); lanes 2 and 7, BL21(DE3)/pJH2; lanes 3 and 8, BL21(DE3)/pJH4; lanes 4 and 9, BL21(DE3)/pJH7; lanes 5 and 10, BL21(DE3)/pJH8.

(B) BL21(DE3)/pJH13, BL21(DE3)/pJH14, and BL21(DE3)/pJH17 were grown and induced as described in (A). Samples (about 20 µg of cellular protein) were heated at 80°C for 5 min prior to analysis by SDS-PAGE. Lane 1, BL21(DE3)/pJH13; lane 2, BL21(DE3)/pJH14; lane 3, BL21(DE3)/pJH17.

Because a major E. coli protein has the same electrophoretic mobility, the protein produced by pJH8 could not be detected by SDS gel electrophoresis. However, using antibody to PE, an immunoblot clearly showed a protein with the expected molecular weight of 40 kd reacting with antibody to PE (Figure 4, lane 5). In comparison with PE standards, the strength of the signal indicated that the 40 kd protein was present at a concentration of approximately 0.04 mg/mg of cell protein. This value has subsequently been confirmed by purification of the 40 kd molecule to near homogeneity (unpublished data). Extracts of pJH8 contained higher amounts of ADP ribosylating activity than predicted by protein analysis, suggesting that the presence of domain Ia may be inhibitory to the enzymatic activity of native PE in this construction. The high ADP ribosylating activity of pJH8 was seen when urea and DTT were not included in the reaction mixture. Whereas the addition of urea and DTT to native PE activated ADP ribosylating activity, the addition of urea and DTT to pJH8 reduced ADP ribosylating activity by about 30%. These data clearly show that the ADP ribosylating domain is contained within structural domains II, Ib, and III.

pJH7

Because of a previous report indicating that the ADP ribosylating activity of PE resided in the carboxy end of the molecule (amino acid residues 492–613; Gray et al., 1984), we constructed plasmid pJH7 which encodes domain III amino acid residues 405 to 613 plus the methionine and three amino acids from the amino terminus of the protein. IPTG-induced cells produced large amounts of a 23 kd protein which shows up as a prominent band on

Table 1. Summary of the Amount and Activity of the Recombinant Toxins Measured by SDS-PAGE, ADP-Ribosylation, and Cell Killing Experiments

	Amount of PE per mg of Cellular Protein				
	Measured by SDS-PAGE (mg)	Measured by ADP-Ribosylation[a] (units)		Measured by Cell Killing[a] (units)	
	Total	Supernatant	Pellet	Supernatant	Pellet
pJH1	0.20[b]	N.D.	N.D.	N.D.	N.D.
pJH2	0.20[b]	N.D.	N.D.	N.D.	N.D.
pJH3	degraded	<0.001	<0.001	N.D.	N.D.
pJH4	0.20[b]	0.10	0.20	<0.001	0.2
pJH5	degraded	0.15	0.02	N.D.	N.D.
pJH6	degraded	<0.001	<0.001	N.D.	N.D.
pJH7	0.25[bc]	<0.001	<0.001	<0.001	<0.001
pJH8	0.04[cd]	0.66	0.04	<0.001	<0.001
pJH9	0.15[a]	0.40	0.20	<0.001	<0.001
pJH10	0.15[a]	0.40	0.15	<0.001	<0.001
pJH11	0.25[a]	<0.001	<0.001	<0.001	<0.001
pJH12	<0.01[cd]	<0.001	<0.001	N.D.	N.D.
pJH13	0.01	0.25	0.20	<0.001	<0.001
pJH14	0.30[a]	<0.001	<0.001	<0.001	<0.001
pJH15	0.10[a]	0.18	0.30	<0.001	<0.001
pJH16	<0.01[d]	0.02	N.D.	N.D.	N.D.
pJH17	0.03[cd]	0.06	<0.001	<0.001	<0.001
pJH18	<0.01[d]	0.02	N.D.	N.D.	N.D.

[a] One unit of ADP-ribosylation or cell killing activity is equivalent to the activity from 1 mg of native PE.
[b] aggregated.
[c] positive by Western.
[d] not visible on SDS-PAGE.
N.D. = not determined.

Table 2. Characteristics of Recombinant Toxins Studied

Construction	Domain Present	Protein Size
pJH4	Met, I, II, III	62 kd
pJH7	III	23 kd
pJH8	II, Ib, III	40 kd
pJH13	I, half of II, III	62 kd
pJH14	Ia	27 kd
pJH17	20 a.a. of Ib, III	26 kd

SDS gel electrophoresis (Figure 3, lane 4). About 0.25 mg of the 23 kd protein is produced per milligram of cell protein; large amounts are found both in the soluble fraction and in the pellet (Figure 5, lanes 1 and 2). To ensure that the protein produced by pJH7 was truly a portion of PE, an immunoblotting experiment was carried out and the 23 kd protein reacted with antibody to PE (Figure 4, lane 4). When extracts containing domain III were further tested in an ADP ribosylation assay, no activity could be detected over background levels (Table 1), and the addition of urea or DTT, substances that can activate native PE (Leppla et al., 1978), did not bring out any activity. This result suggests that domain III produced by pJH7 does not contain the enzymatically active domain. We also constructed plasmid pJH9, which contains amino acid residues 492 to 613, and were also unable to detect ADP ribosylating activity in extracts of cells expressing this plasmid.

pJH17

We next constructed plasmid pJH17, which contains domain III and 20 amino acids from domain Ib. Extracts from this construction contained high levels of ADP ribosylating activity equivalent to 0.06 mg PE per milligram of cell protein (Table 1). By immunoblotting a 26 kd and a 23 kd protein were detected and these were present at a concentration of 0.03 mg/mg of cell protein (Figure 4, lane 6). The 23 kd protein may be a degraded product of the 26 kd protein. This result indicates that the ADP ribosylating activity is present at the carboxyl terminus of the molecule but requires more than structural domain III.

pJH14

When this clone, encoding only domain Ia, was expressed, a 27 kd protein was produced in large amounts and was mainly present in the pellet (Figure 3B, lane 2); this protein reacts strongly with antibody to PE. No ADP ribosylating activity was detected when this material was solubilized and assayed.

pJH13

Cells containing this recombinant plasmid, which has a deletion of the first half of domain II, accumulated at about 0.1 mg toxin per milligram of cell protein (Figure 3B, lane 1). The ADP ribosylating activity was high, and present in both the supernatant and pellet (Table 1).

All together, the data from the analysis of pJH8, pJH7, pJH14, and pJH13 indicate that the region responsible for ADP ribosylating activity consists of domain III and part of the adjacent domain Ib.

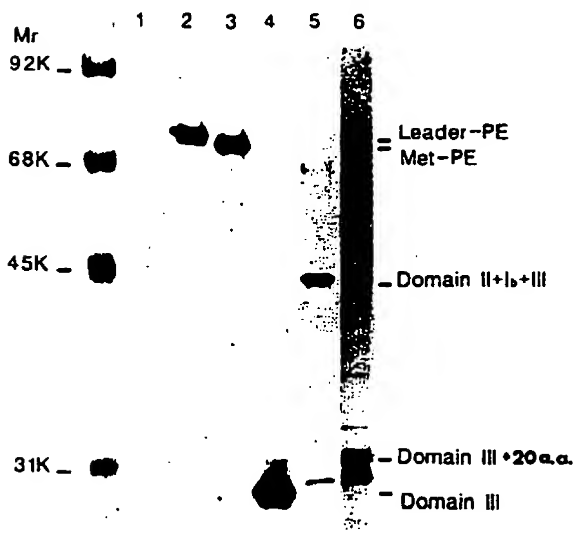

Figure 4. Identification of the Recombinant PE Molecules by Immunoblotting with Antibody to PE

Cells were grown and induced as described in the legend to Figure 3. Samples (about 0.5 μg of cellular protein) in Laemmli buffer were heated at 80°C for 5 min prior to application to a 10% polyacrylamide slab gel. After electrophoresis, samples were transferred to nitrocellulose paper for immunoblotting. Lane 1, BL21(DE3); lane 2, BL21(DE3)/pJH2; lane 3, BL21(DE3)/pJH4; lane 4, BL21(DE3)/pJH7; lane 5, BL21(DE3)/pJH8; land 6, BL21(DE3)/pJH17.

Figure 5. The Distribution of the Recombinant Toxins in Supernatant and Pellet after Sonication

Cells were grown and induced with IPTG as described in the legend to Figure 3. Cells were harvested by centrifugation at 8,000 × g, 4°C for 10 min. Cell pellets were resuspended in 10 mM Tris (pH 8.1) and sonicated for 30 sec three times in an ice bath. After sonication, extracts were centrifuged at 12,000 × g, 4°C for 15 min. Samples from supernatant and pellet were analyzed by SDS-PAGE. Lanes 1, 3, and 5 are from supernatant. Lanes 2, 4, and 6 are from pellet. Lanes 1 and 2, BL21(DE3)/pJH7; lanes 3 and 4, BL21(DE3)/pJH8; lanes 5 and 6, BL21(DE3)/pJH4.

Table 3. Effect of Various PE domains on Reversing Inhibition of Cell Protein Synthesis by Native PE

Domain Tested	^{3}H-Leucine in Corporation (cpm × 10^3) Without PE	With PE (I, II, III)
none	11.2 ± .1	2.3 ± .2
Ia	11.5 ± .15	9.4 ± .3
II, Ib, and III	11.7 ± .15	2.4 ± .2
I, 1/2 II, III	10.7 ± .15	9.2 ± .2
8 M urea (10 µl)	11.1 ± .1	2.9 ± .2

Structural domain Ia (pJH14) and structural domain I, 1/2 II, and III (pJH13) were extracted from the pellet of the sonicated cells with 8M urea. Ten microliters of each extract equivalent to 3–5 µg of recombinants was used in each assay. Structural domains II, Ib, and III (pJH8) were present in the supernatant of the sonicated BL21(DE3)/pJH8 cells. Ten microliters of extract equivalent to 2 µg of recombinant toxin was used. Cells were treated as indicated for 15 min with and without native PE at 0.1 µg/ml followed by 1 ml DMEM washing, incubated 4 hr in DMEM + 0.2% BSA, and then incubated with ^{3}H-leucine for 1 hr.

Cell Binding Domain

It is well established that PE binds to cells as the first step in cell killing, although the cellular receptor for PE has not yet been identified (Eidels et al., 1983; Middlebrook and Dorland, 1984). Since specific binding is saturable, it should be possible to identify portions of the PE molecule that are capable of binding to the receptor and thereby, when present in excess, prevent PE binding by competition. Since PE binding results in inhibition of protein synthesis and cell death, molecules which prevent binding will prevent these toxic effects of PE.

None of the recombinant toxins that contained deletions of different regions of PE had significant cell killing activity, even though many of these had high enzymatic activity (Table 1). Since these molecules were not toxic to cells, we tried to determine whether they could prevent inhibition of protein synthesis or cell killing by native PE. To do this, cells were exposed for 15 min to PE at 0.1 µg/ml in the presence of 3–5 µg/ml of various modified toxins. The data in Table 3 show that constructions expressing either domain Ia alone, or domain I, half of domain II, and domain III prevent PE from inhibiting protein synthesis. However, a construction expressing domain II, Ib, and III without domain Ia did not. (Since in some cases 8 M urea was used to solubilize the modified toxins, it was tested and found not to affect the assay.) These results indicate that domain Ia of PE contains the cellular binding site.

Met-PE produced by pJH4, like native PE, was fully capable of inhibiting protein synthesis and cell killing. Cell killing activity was present only when Met-PE was extracted from the pellet (Figure 5, lane 6); the material in the supernatant was proteolytically degraded and had apparently lost its cell binding domain (Figure 5, lane 5).

Animal Toxicity

To compare the animal toxicity of native PE with PE containing a deletion of domain Ia, PE(ΔIa), which was produced by plasmid pJH8, the recombinant material was purified to about 95% homogeneity by gel filtration and ion exchange chromotography (Hwang et al., unpublished data). This material and native PE were injected into mice in groups of three (Table 4). All mice receiving 20 µg of PE(ΔIa) or less survived whereas two of three mice receiving 50 µg PE(ΔIa) died. In contrast, one of three mice receiving 0.2 µg of native PE, and all mice receiving 0.3 µg PE or more, were killed. Therefore PE(ΔIa) is about 200-fold less toxic than PE.

Table 4. Animal Toxicity of a Recombinant Toxin with Deletion of Domain Ia

Toxin	Dose (µg)	Deaths
PE(ΔIa)	50	2/3
PE(ΔIa)	20	0/3
PE(ΔIa)	5	0/3
PE	10.0	3/3
PE	1.0	3/3
PE	0.3	3/3
PE	0.2	1/3

BALB/c mice were injected intraperitoneally with various amounts of PE or PE (ΔIa) contained in 1.0 ml of sterile saline with 10 mg/ml human serum albumin. The animals were monitored daily for 2 weeks. All deaths occurred at 48 hr.

Discussion

Pseudomonas exotoxin A (PE) is the most toxic of the extracellular proteins produced by Pseudomonas aeruginosa (Liu, 1966). This toxin is active on most eukaryotic cells and species, including humans (Thompson and Iglewski, 1982). The intoxication process is thought to proceed by the following steps. First, PE binds to cells through a specific receptor on the cell surface. Next, the PE–receptor complex is internalized into the cell. Finally, PE is translocated to the cytosol where it enzymatically inhibits protein synthesis by inactivation of elongation factor 2 (Eidels et al., 1983; Middlebrook and Dorland, 1984). The translocation process is thought to occur in an acidic environment since cellular intoxication is prevented by weak bases such as NH_4^+, which raise the pH in acidic vesicles (Michael and Saelinger, 1979; FitzGerald et al., 1980). It has been suggested that upon exposure to acidic conditions, the hydrophobic domain of PE inserts into the membrane, resulting in the formation of a channel through which the enzymatic domain, in extended form, can pass into the cytosol (Kagan et al., 1981; Sandvig and Olsnes, 1981).

Recently PE has been crystallized and its three-dimensional structure determined (Allured et al., 1986). The molecule is composed of three structurally distinct domains. We have studied the structure–function relationships of PE domains and, assuming no distortion of the structure of the individual domains in partial PE molecules, have been able to assign different functions to the three structural domains (Figure 2). The three functions are binding to cells, translocation across a membrane, and enzymatic activity.

Cell Binding Domain

In this study, we found that molecules containing structural domain Ia (pJH14) blocked cytotoxic activity of intact

PE on sensitive cells, whereas molecules lacking structural domain Ia and containing structural domains II, Ib, and III (pJH8) failed to block PE cytotoxicity. These results suggest that the binding activity of PE is located within structural domain Ia. In addition, PE with a deletion of part of domain Ia (amino acids 4 to 134; pJH15) was a very poor cell killing agent, at least 1000-fold less than native PE. This result is also consistent with the notion that binding of PE is located within structural domain Ia.

Translocation Domain

PE molecules with a deletion of the first half of structural domain II (pJH13) exhibited both PE blocking activity and ADP ribosylation activity, indicating that the binding and enzymatic domains are intact. Nevertheless, this construction had lost all cell killing activity. This result suggests that the first half of structural domain II is required for translocation of PE across a membrane from an endocytic vesicle into the cytoplasm. An examination of the amino acid sequence of this region shows that the first half of structural domain II contains a very hydrophobic domain which could interact with lipids present in the membrane of endocytic vesicles (Gray et al., 1984; Allured et al., 1986). However, until direct evidence that domain II possesses translocation activity is obtained by fusing domain II to other molecules that do not ordinarily translocate across membranes, the notion that domain II acts in a different manner is possible.

ADP Ribosylation Region

Previously, Gray et al. (1984) reported that structural domain III contained ADP ribosylating activity although the level of activity measured was low. When we expressed a plasmid (pJH7) that encodes only structural domain III, large amounts of a protein of the expected domain III size, i.e., 23 kd, were produced. However, when we examined ADP ribosylation activity, we were unable to detect significant enzymatic activity. This suggested that the enzymatic function might require more than structural domain III. We therefore constructed a plasmid that encodes structural domain III and 20 adjacent amino acids from domain Ib (pJH17). The molecule produced by pJH17 contains the largest PE segment, which does not contain cystine residues. This fragment of PE was expressed at a level of 3% of total cellular protein and its ADP ribosylation activity was high. This result shows that structural domain III requires adjacent amino acid residues for its ADP ribosylation activity. The minimum length of the amino acid chain required has not been determined. It has been previously reported (Chung and Collier, 1977; Lory and Collier, 1980) that one can obtain an enzymatically active fragment of PE that lacks cystine residues; no cystine residues are present in the protein produced by pJH17. A 40 kd partial PE molecule containing structural domains II, Ib, and III also exhibited very high specific ADP ribosylation activity. This observation may indicate that after removal of a portion of the molecule distant from the ADP ribosylating site, PE in some way becomes more accessible to EF-2 and NAD, resulting in enhanced ADP ribosylation activity. Results with pJH13 are in agreement with this interpretation (Table 1).

We have constructed a total of 18 plasmids that encode different portions of PE. The results with other plasmids, also described in Table 1 and Figures 1 and 2, are consistent with the results already discussed.

Animal Studies

When administered to animals, PE characteristically produces death by liver failure (Saelinger et al., 1977; Pavlovskis et al., 1978). Immunotoxins made with PE also attack the liver and, when given in large amounts, produce death due to liver toxicity (FitzGerald, Willingham, and Pastan, unpublished result). The experiments shown in Table 3 indicate that domain Ia is responsible for cell binding and suggest that a PE molecule in which domain Ia is deleted PE (ΔIa) should be less toxic to mice than native PE. The data of Table 4 show that this is so and that PE (ΔIa) is about 200-fold less toxic than native PE. Thus PE molecules with a deletion of domain Ia should make effective immunotoxins with diminished liver toxicity. The ability to produce large amounts of PE with a deletion of domain Ia will allow the study of these possible immunotoxins.

Experimental Procedures

Materials

Restriction endonucleases, S1 nuclease, T4 DNA ligase, and enzymes used in cloning DNA were obtained from New England Biolabs or Bethesda Research Laboratories and used under conditions recommended by the supplier. Synthetic deoxynucleotide linkers were prepared at the National Cancer Institute. Nicotinamide [U-^{14}C] adenine dinucleotide, ammonium salt [^{14}C-NAD) was purchased from Amersham Corporation. Immunoblotting kits were bought from Vector Laboratories, Inc. Reagents for SDS-PAGE were obtained from Bio-Rad. All other chemicals are analytical reagent grade.

Bacteria Strains and Plasmids

E. coli strain HB101 (F$^-$ recA 13, hsdR, hsdM, lacY1 supE44, ara, proA, galK, xyl, mtl, rpsL), from Bethesda Research Laboratories, was used as host for most experiments (Boyer and Roulland-Dussonix, 1969). P. aeruginosa strain PA103 (Leppla, 1976) was used for Pseudomonas chromosomal DNA preparation. pUC13 was obtained from Boehringer Mannheim. BL21(DE3), which carries T7 RNA polymerase gene in lysogenic and inducible form (Studier and Moffatt, 1986), was used as host for recombinant PE expression. pAR2156 (Studier and Moffatt, 1986), which carries the T7 late promoter and Amp^r, was used as vector for the PE structural gene. Both BL21(DE3) and pAR2156 were gifts from F. W. Studier, Biology Department, Bookhaven National Laboratory, Upton, NY.

Isolation of PE Structural Gene and Construction of PE Expression Plasmids

Chromosal DNA prepared from our laboratory isolate of P. aeruginosa strain PA103, which produces large amounts of active toxin, was digested with EcoRI and PstI. DNA fragments ranging in size from 2.6 to 2.9 kb were isolated and ligated with a 2.7 kb pUC13 DNA fragment which was also cut with EcoRI and PstI. Competent HB101 cells were then transformed with the ligation mixture. The 2.7 kb DNA fragment derived from ptoxETA (Gray et al., 1984), which contains PE structural gene, was used as probe in colony hybridization. Plasmid DNA from several positive colonies was prepared and characterized by Southern blotting. One such clone is called pEO (see Figure 1). To express different portions of PE in the BL21(DE3) host, derivatives of pAR2156 with

the T7 late promoter and a Shine-Dalgarno fox fused to different portions of the PE structural gene were constructed as follows.

pJH1: pEO DNA was partially cut with BamHI and the linear form of DNA was eluted and completely cut with EcoRI. The 2.0 kb DNA fragment derived from PEO, which has BamHI and EcoRI at both ends, was inserted into pAR2156, which had been completely cut with BamHI and EcoRI. The plasmid (pJHI) was examined for the size of the DNA (6.0 kb) and its ability to express PE.

pJH2: pJH1 was partially cut with BamHI. The linear form of DNA was isolated and completely cut with NdeI. The 6.0 kb DNA fragment was used to construct pJH2 by ligation with the synthetic oligonucleotide duplex.

$^{5'}$TATGAAAAA
ACTTTTTCTAG$^{5'}$

The plasmid (pJH2) was examined for the size of the DNA (6.0 kb) and for PE expression.

pJH4: pJH2 was partially cut with TaqI. The linearized DNA (6.0 kb) was isolated and cut with NdeI. The largest DNA fragment (5.9 kb) was ligated to a synthetic oligonucleotide duplex which contained

$^{5'}$TATGGCCGAAGAAGCTTT
ACCGGCTTCTTCGAAAGC$^{5'}$

a HindIII site (AAGCTT). The plasmid pJH4 was examined for the size of the DNA (5.9 kb) and PE expression. In this new plasmid (pJH4) we created a HindIII site, but we did not change the amino acid sequence.

pJH7: pJH4 was partially cut with AatII. The linearized DNA fragment (5.9 kb) was completely cut with HindIII. The 4.7 kb DNA fragment, which has AatII and HindIII sites at its ends, was incubated with S1 nuclease to remove the cohesive ends followed by ligation with T4 DNA ligase. The plasmid (pJH7) was then examined for size (4.7 kb) and for its ability to express domain III of PE.

pJH8: pJH4 was partially cut with AvaI. The linearized DNA fragment was then completely cut with HindIII. The 5.1 kb DNA fragment, which has AvaI and HindIII sites at its ends, was incubated with S1 nuclease to remove cohesive ends followed by ligation with T4 ligase. The plasmid (pJH8) was then examined for DNA size (5.1 kb) and for its ability to express domains II, Ib, and III of PE.

pJH13: pJH4 was partially cut with AvaI. The linearized DNA fragment was then completely cut with EcoRI. The 4.7 kb DNA, which has AvaI and EcoRI sites at both ends, was incubated with S1 nuclease to remove cohesive ends (DNA fragment 1). pJH4 was partially cut with SalI. The linearized DNA fragment was then completely cut with EcoRI. The 1.0 kb DNA fragment, which has SalI and EcoRI sites at the ends, was incubated with Klenow DNA polymerase I and dNTP to fill the cohesive ends (DNA fragment 2). DNA fragment 1 (4.7 kb) and DNA fragment 2 (1.0 kb) were ligated at 4°C overnight. The plasmid pJH13 was examined for its size (5.7 kb) and for its ability to express domain I + 1/2 II + III of PE.

pJH14: pJH4 was partially cut with AvaI. The linearized DNA fragment was then completely cut with EcoRI. The 4.7 kb DNA, which has AvaI and EcoRI sites at its ends, was incubated with S1 to remove cohesive ends followed by ligation with T4 ligase. The plasmid pJH14 was then examined for DNA size (4.9 kb) and for its ability to express domain Ia of PE.

pJH17: pJH4 was completely cut with HindIII and ApaI. The 4.8 kb DNA fragment was isolated and incubated with Klenow DNA polymerase I and dNTP to fill the cohesive ends followed by ligation with T4 DNA ligase. The plasmid pJH17 was examined for size of the DNA (4.8 kb) and for the ability to express domain III with adjacent 20 amino acids of PE.

Expression of the Recombinant Toxins in BL21(DE3)

For expression of PE, BL21(DE3)-containing plasmids were cultured in LB broth with 50 µg per ml ampicillin at 37°C. When absorbance at 650 nm reached 0.3, IPTG was added at a final concentration of 1 mM. Cells were harvested 120 min later and analyzed for the amount of recombinant toxin produced by SDS-PAGE, immunoblotting, ADP ribosylation assays, and cell killing experiments.

SDS-PAGE and Immunoblotting

Cell pellets were dissolved directly in Laemmli buffer (Laemmli, 1970). Samples were boiled for 5 min prior to application to a 0.1% SDS, 10% acrylamide slab gel and stained by Coomassie blue after electrophoresis as described by Laemmli (Laemmli, 1970). For immunoblotting, electrophoresed samples were transferred from gels to a nitrocellulose paper, followed by reaction with antibody to PE, then second antibody (goat anti-rabbit) and then staining. Procedures were carried out under conditions recommended by the supplier (Vector Laboratories, Inc.). The antibody to PE was obtained by hyperimmunizing rabbits with PE treated with 0.2% glutaraldehyde (250 µg of PE per injection). An IgG fraction was prepared for immunoblotting.

Assay of ADP Ribosylation Activity

For an assay of ADP ribosylation activity, the general procedure of Collier and Kandel was followed (1971). A rabbit reticulocyte preparation or wheat germ extracts enriched for elongation factor 2 (EF-2) was used as a source of EG-2. Assays (500 µl total volume) contained about 10 pmole of EF-2, 37 pmole of ^{14}C-NAD (0.06 µCi), 0.25 to 1.25 µg of PE, and buffer (40 mM DTT, 1 mM EDTA and 50 mM Tris [pH 8.1]). Activity was measured as pmoles of NAD transferred to EF-2 in 30 min. A standard curve of known concentrations of PE was established and used to determine the activity of PE in extracts from E. coli. After incubation for 30 min 37°C, 0.5 ml 12% TCA was added to each new assay mixture. The assay mixtures were then set in an ice bath for 15 min, followed by centrifugation at 4°C, 3,000 × g for 10 min. The pellet was washed with 1 ml 6% TCA and centrifuged as above. The ^{14}C content of the pellet was measured in a liquid scintillation counter as an index of the ADP ribosylation activity.

Cell Cytotoxicity Test

Tests of the cytotoxic activity of exotoxin were performed in NIH 3T3 cell cultures. NIH 3T3 cells were seeded 24 hr prior to the cytotoxicity test in a 24-well tissue culture plate at a density of 2×10^4 cells per well. After incubation for 48 hr with various concentrations of PE or extracts of BL21(DE$_3$) expressing recombinant plasmids, the monolayers were stained with methylene blue to detect the surviving cells (Table 1).

Inhibition of Protein Synthesis

Assays for the inhibition of protein synthesis by PE alone or PE with extracts of BL21(DE3)/pJH8, BL21(DE3)/pJH13, and BL21(DE3)/pJH14 were performed in Swiss 3T3 cell cultures. Swiss 3T3 cells were seeded one day before assay in 24-well tissue culture plates at a density of 10^5 cells per well. Cells were washed once by replacing medium with DMEM containing 0.2% BSA before adding PE (100 ng/ml) alone or PE (100 ng/ml) and extracts of BL21(DE3) expressing different portions of PE (Table 2). After 15 min at 37°C, the medium were removed and replaced with fresh DMEM containing 0.2% BSA. Four hours later, [^{3}H] leucine was added to the medium at a final concentration of 2–4 µCi/ml and cells incubated for 1 hr to measure the rate of protein synthesis.

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